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The Role of Tyrosines in Elastase

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The conformational behavior of elastase between pH 7 and 11 has been investigated, with particular emphasis on tyrosine residues. Of these, ten titrate normally and one is totally inaccessible to ionization. On the basis of chemical modification, the eleven tyrosines can be subdivided into three reactive classes and one totally unreactive. The first class, consisting of eight groups, can be cyanurated and is easy to acetylate; the other two classes consist of one group each, which can be acetylated with increasing difficulty but cannot be cyanurated. The eleventh residue is inaccessible to either reagent. Circular dichroism studies showed that the secondary structure remains invariant between pH 5 and 11, since the far uv CD spectrum remains unchanged. The fact that acetylation of ten tyrosines neither affects the far uv CD spectrum nor the enzymatic activity rules out the structural stabilization of the active enzyme by a tyrosine. Circular dichroism studies at higher wavelengths indicate that the alkaline transitions found in chymotrypsin do not occur in elastase.

Elastase is a serine proteinase which is homologous to chymotrypsin; the two enzymes have similar tertiary structures (1–3) and identical catalytic activities and mechanisms of action (4–6). Both contain an internal ion pair, Ile-16-Asp-194 in chymotrypsin, and Val-16-Asp-194 in elastase. In chymotrypsin, this ion pair is a key to the formation and maintainance of the active conformation of the enzyme (7); it does not appear to play a similar role in elastase. Thus, in chymotrypsin both acetylation (8) and deprotonation (9) of Ile-16, which preclude the formation of this ion pair, result in loss of enzymatic activity and in a conformational change; a similar situation is not true for the NH_2-terminal Val-16 of elastase.

It has been reported that when all the amino groups of elastase are fully acetylated, the enzyme retains complete activity; also the pH activity curves for methyl esters of *N*-benzoyl-L-alanine and acetyl-L-alanyl-L-alanyl-L-alanine do not reflect the ionization state of Val-16 (10, 11). The large pH-dependent change in optical rotation which exists in chymotrypsin (8, 9) has not been found in the case of elastase (12). A group with a pK of ~10.5, however, has been implicated in the control of the enzymatic activity of elastase (4). The nature of this group is unclear, although a suggestion has been made that it might be a tyrosine (13) and that the structural transition of elastase above pH 10.5 is related to the ionization of a tyrosine residue (14).

This study was undertaken in the hope of gaining insight into the conformational behavior of elastase in the alkaline region and

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of establishing the state of the tyrosine residues and their possible contribution to the conformational stability of the active enzyme. This paper reports the results of circular dichroism studies of elastase in the pH range between 5 and 11 and of the titration and chemical modifications of tyrosine residues with *N*-acetylimidazole and cyanuric fluoride.

MATERIALS AND METHODS

Elastase was prepared according to the method of Shotton (15) from Trypsin 1-300 purchased from Nutritional Biochemicals Corp.[2] (Lots #3871 and 7756). *N*-Benzyloxycarbonylglycine *p*-nitrophenyl ester was obtained from Sigma Chemical Co. The other reagents used were as described before (16). Spectroscopic measurements were made at room temperature on a Cary Model 14 recording spectrophotometer. The pH values were measured at room temperature with a Radiometer 28 pH meter. Protein concentrations were determined spectrophotometrically in 0.01 M Na acetate (pH 5), using a molar extinction coefficient at 280 nm of 5.23×10^4 (15).

Spectrophotometric titrations were carried out by the difference spectral technique (17) in 1 M $KHCO_3$ buffer with and without 10% dioxane. The uv spectra were recorded against protein solutions dissolved in 0.01 M acetate buffer (pH 5) with and without 10% dioxane. The optical density within 2 min after mixing was taken as the final value. The maxima of the difference spectra were found to be at 297.0 nm. The number of ionized tyrosine residues was calculated using an extinction coefficient for the phenoxide ion of 2300 at 295 nm (18, 19).

Reaction with CyF[3]

The procedure used was the same as described previously (20) except that the cyanuration was carried out at 4°C and the reaction time was limited to 45 min. At the completion of the reaction, the pH of the reaction mixture was adjusted to 13.0. The uv spectrum was then recorded in 2-cm cells between 290 and 340 nm after 2 min at pH 13.0 against a standard of the same concentration in 0.01 M acetate buffer (pH 5) which contained 10% dioxane. Since it was found that CyF-treated protein adjusted to pH 7 showed a weak positive absorption at 297.0 nm relative to untreated protein dissolved in the 0.01 M acetate buffer (pH 5) with 10% dioxane, the extent of this absorption was determined for all pH values used in cyanuration experiments. The values so obtained were then introduced into the calculations as blank corrections (20).

The number of moles of tyrosine residues which had reacted under any given conditions of pH and CyF concentration was calculated as before (20). The uv spectra at pH 13 of the cyanurated protein were obtained after 2 min because CyF-treated elastase developed turbidity on standing at that pH. Since, however, spectrophotometric titration had shown that, under these conditions, only ten out of 11 residues are ionized, the absorption values obtained at 2 min were corrected by dividing them by 10/11. The data were analyzed according to the previously described procedure (16).

Acetylation

Acetylation with *N*-acetylimidazole was carried out at pH 7.5 according to the method of Riordan *et al.* (21), except that the reaction was conducted at 4°C, the reagent concentration was varied and chromatography was replaced by dialysis in 0.01 M acetate buffer (pH 5). The elastase concentration necessary for calculation of the results was estimated in the following way. In control experiments, it was found that exposure of elastase to the reaction conditions of acetylation including dialysis resulted in a protein recovery of only 90–96%, compared to concentrations obtained by direct dilution of the same stock solution in 0.01 M acetate buffer (pH 5). Therefore, every acetylated sample was treated with hydroxylamine at pH 7.0 to reverse acetylation (22). The absorbance at 280 nm, which was measured after 15 min (the optical density increased only during the first 10 min), was assumed to represent the concentration of the regenerated elastase. Values obtained in this way corresponded to 93–95% recovery when compared to concentrations given by direct dilutions of the stock solution, the same as in the control experiments.

Circular Dichroism

The circular dichroism (CD) experiments were carried out on a Cary Model 60 spectropolarimeter equipped with a Model 6001 CD attachment, as described previously (16). Protein concentrations were adjusted to an absorptivity of approx 1.7 at 280 nm. Each experiment was carried out in three cells of different optical path lengths, depending on the particular spectral region of interest. A 1.0-cm cell was used between 350 and 240 nm; the

[2] Mention of companies or products is for the convenience of the reader and does not constitute an endorsement by the U. S. Department of Agriculture.

[3] Abbreviations used are: CD, Circular dichroism; CyF, cyanuric fluoride; TNBS, 2,4,6-trinitrobenzene sulfonic acid.

cell thickness was reduced to 0.1 cm for the experiments between 260 and 200 nm; and a 0.01-cm cell was used in the far uv, from 250 to 185 nm. All the cells were jacketed. The temperature was maintained at 2°C by circulating a water–alcohol mixture from a constant-temperature bath controlled at 2 ± 0.05°C. The results were calculated as $[\theta]$, the ellipticity in degrees-cm² per decimole of residue, without introduction of the Lorenz refractive index factor. The mean residue weight was calculated to be 108.0.

Enzymatic Assays

The enzymatic activity of elastase was determined spectrophotometrically at 400 nm and pH 8.2 using *N*-benzyloxycarbonyl-glycine *p*-nitrophenyl ester (23, 24).

Determination of the Number of Labile Acetyl Residues

Elastase, which had been acetylated with *N*-acetylimidazole, was treated with a 2 M hydroxylamine solution for 10 min at either pH 11.6 (25) or pH 7.0 (22) to determine the number of labile acetyl residues.

Autolysis

Elastase (0.5 ml) dissolved in 0.01 M acetate buffer (pH 5) was mixed with 2 ml of 0.01 M buffer of a given pH and left for 5 hr at a given temperature. The mixture was transferred then to a dialysis bag, the reaction flask was washed out twice with 0.25 ml 0.01 M acetate buffer (pH 5) and the combined solution was dialyzed in 50 ml of 0.01 M acetate buffer (pH 5) overnight at 4°C.

RESULTS

Since the CD experiments take several hours and there was insufficient information on the stability of elastase in the alkaline range (12, 15, 26), elastase was incubated for 5 hr in the pH range from 7 to 12 at 4 and 25°C. The results of these experiments are compiled in Table I. The pH 5 data are included, since they are used as a reference point. Both the percentage of recovery and of activity were calculated relative to the pH 5 sample, made up from the same stock solution by appropriate dilution, with the assumption that the activity of the pH 5-incubated sample (see experimental) was 100%.

As can be seen from Table I, there is a striking correspondence between recovery and remaining activity, suggesting that the observed inactivation is directly related to autolysis. At 4°C the enzymatic activity of elastase is not affected up to pH 11.0, but decreases to 50% at pH 11.6. At 25°C there is a rapid decrease in activity with increase in pH above 8. Exposure to pH 12.1 resulted in precipitation of the protein during dialysis. Furthermore, the uv spectra of the elastase samples autolyzed at 4°C in the pH range from 7 to 11 were identical to the uv spectrum of the pH 5 starting material. These spectra were characterized by a doublet with maxima of equal extinction coefficients at 276 and 282 nm, as previously described by Visser and Blout (27). The uv spectra of elastase samples which had been incubated at 25°C in the pH range from 7.8 to 9.8 showed an increase in the magnitude of the 276-nm band and a gradual decrease of the intensity at 282 nm. The same phenomenon was observed for some of the samples which had been exposed to pH 7.0 at 25°C for a long time, although their activity remained at 100%, as well as for those exposed to pH 11.6 at 4°C.

TABLE I

AUTOLYSIS OF ELASTASE[a]

pH	25°C		4°C	
	% Recovery[b]	% Activity[c]	% Recovery[b]	% Activity[c]
5.0	95.2	(100)	96.0	(100)
7.0	92.0	100	95.4	100
7.8	94.4	80	96.3	100
8.5	79.0	73	94.6	100
9.8	59.5	60	94.9	100
10.5	52.6	51	95.7	100
11.0	45.0	33	92.0	100
11.6	32.1	0	53.0	100
12.1	Turbid	0	—	50

[a] Exposure for 5 hr to given conditions of pH and temperature.

[b] Percentage of recovery determined by measuring the optical density at 280 nm after the dialysis step and comparing it to the optical density of an undialyzed reference solution in 0.01 M acetate buffer (pH 5).

[c] The activity of a sample incubated for 5 hr at pH 5 and dialyzed was taken as 100%.

Titration

Elastase was titrated spectrophotometrically at 25°C between pH 8.5 and 13.0 in 1 M

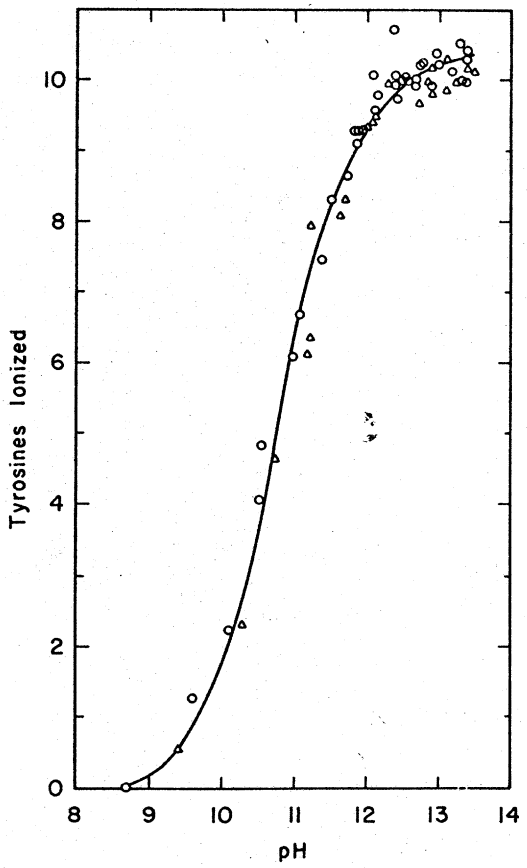

FIG. 1. Spectrophotometric titration of elastase tyrosines.

$KHCO_3$ buffer both in the presence and absence of 10% dioxane. Addition of 10% dioxane to elastase dissolved in 0.01 M acetate buffer (pH 5) resulted in the perturbation of both tyrosine and tryptophan residues, although the titration curves were essentially identical. The solvent perturbation-difference spectrum was characterized by a large maximum at 286 nm and a smaller one at 293 nm and was similar to that reported by Visser and Blout (27) for 20% glycerol.

The results of the titration are shown on Fig. 1. Above pH 13, the data became unreliable. There was a rapid development of turbidity and probably also breakdown of the protein, since the apparent extent of ionization decresed with an increase in pH. The data can be described best in terms of ten groups ionizing with a pK_{approx} of 10.7 and one group remaining unionized even at pH 12.5.

Circular Dichroism

The results of the circular dichroism experiments are presented in Figs. 2 and 3.

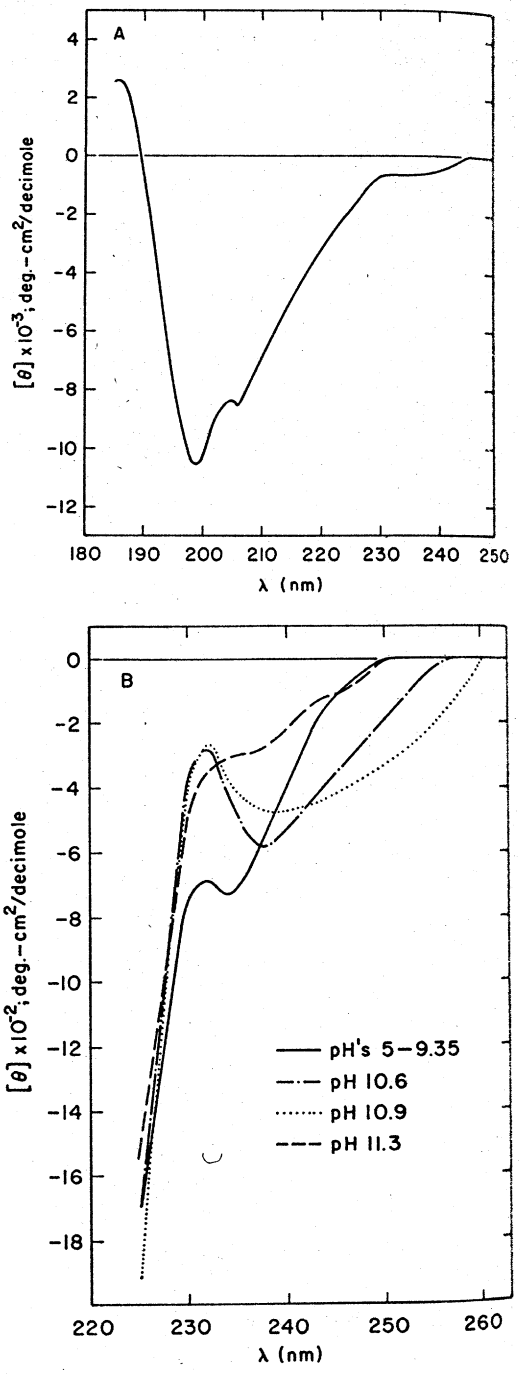

FIG. 2. Circular dichroism spectra of elastase; (a) far uv region, average of 14 runs between pH 5 and 11; (b) middle uv region.

Because of the rapid autolysis at 25°C, all CD experiments were carried out at 2°C. Figure 2a is the far uv spectrum which remained unchanged within experimental error between pH 5 and 10.9. Spectra taken at 25°C and pH 7, both in the presence and absence of 10% dioxane, were identical with those at low temperature. The reported spectrum represents the average of 14 experiments. While similar to the CD spectra of the chymotrypsins in general features, it nevertheless shows important differences in detail. The negative extremum, which appears at 202 nm in α-chymotrypsin (28), is shifted to 199 nm in elastase. Furthermore, as also reported by Visser and Blout (27), a second weaker negative extremum appears at 206 nm. Above this wavelength the intensity decreases monotonely up to 230 nm, where a weak shoulder is observed. Below 199 nm, the ellipticity again becomes rapidly less negative, passes a cross-over point at 189 nm and appears to reach a weak positive peak at 186 nm. The strong negative band at 216 nm, which is characteristic of the chymotrypsin spectrum, is absent from that of elastase, and the spectrum, in general, resembles more that of chymotrypsinogen than of chymotrypsin.

Because of the correlation of CD spectral changes at 220–230 nm with the activation of chymotrypsin, this region was examined in detail. The results are shown in Fig. 2b. The solid line represents the average of 11 experiments between pH 5 and 9.3, over which pH range the spectrum remains invariant. Like the chymotrypsin system, this region of the spectrum is characterized by the presence of a weak band (9). In native elastase, this manifests itself as a negative peak at 234 nm, which most probably corresponds to a weak band at 235 nm, with an amplitude of ca −250 deg-cm²/decimole. Alternately, this could be the result of a somewhat stronger positive band centered near 230 nm, with an ellipticity of ca +400 deg-cm²/decimole. Increase in pH to 10.5 red shifts this band toward 238 nm, while the negative minimum at 232 nm remains unshifted, only decreasing in ellipticity by about a factor of two. At a still higher pH (11.3) the negative extrema in this region essentially vanish with only some

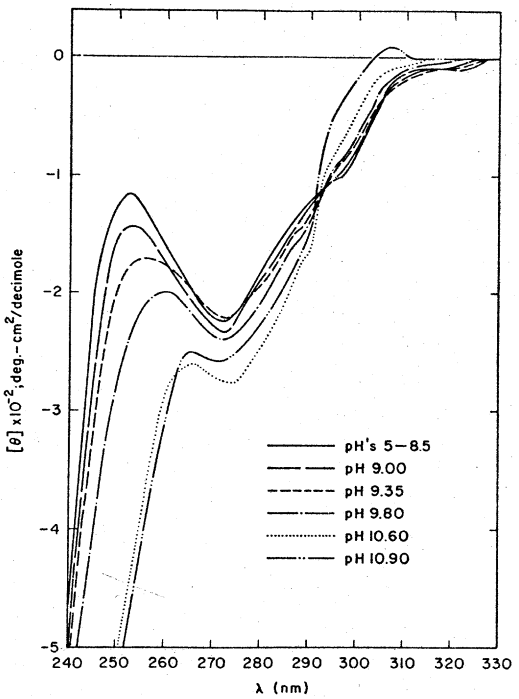

FIG. 3. Circular dichroism spectra of elastase in the near uv region.

weak ellipticity remaining. It is noteworthy that this sharp change in the CD spectrum occurs in the same pH region as that in which autolysis sets in (see Table 1).

The near uv CD spectrum of elastase is shown in Fig. 3. Once again, no changes occur between pH 5 and 8.5. The solid line, which is the average of nine experiments, represents the spectrum of the native enzyme. The predominant feature of this spectrum, which is totally different from that of the chymotrypsins (28), is a negative band at 273 nm. Above this wavelength, shoulders appear at 295 and between 310 and 320 nm. Below 273 nm the ellipticity becomes less negative, reaches a minimum at 252.5 nm and then again rapidly increases in negativity. As the pH is raised above 8.5, the negative minimum becomes weaker and gradually shifts to higher wavelengths while the amplitude of the 273-nm peak gradually increases with only a slight red shift in its position. At the same time, the ellipticity above 292 nm gradually becomes less negative, until at pH 10.9 a positive peak appears at 307 nm. In fact, an isobestic point is quite evident at 292 nm.

Acetylation

Elastase was acetylated in 0.05 M borate buffer (pH 7.5) according to Riordan *et al.* (21), but at 4°C to preclude autolysis and denaturation. The results of acetylation are shown in Fig. 4. For molar ratios of protein to *N*-acetylimidazole between 65 and 430, acetylation affects from four to seven tyrosine residues; a 520-molar excess is required for the acetylation of the eighth residue, while that of the ninth residue requires up to a 950-fold excess. The tenth residue can be acetylated with a very large excess of the reagent but only with great difficulty. The last residue is totally resistant to acetylation.

Acetylation of ten tyrosine residues did not seem to affect the structure of the enzyme to any significant extent. First, the enzymatic activity of elastase was not altered by the acetylation of ten tyrosines. Second, the far uv CD spectrum of elastase with ten groups acetylated was essentially indistinguishable from that of the virginal enzyme, indicating that no serious changes had occurred in the secondary structure.

The higher wavelength CD spectra are affected in a revealing manner by acetylation, as shown in Fig. 5. The CD spectra in both the 230 and 275 nm regions are not affected by the acetylation of seven tyrosines. Acetylation of the next two to three groups produces changes in both spectral regions. Thus, in the near uv, the CD spectrum of highly acetylated elastase remains qualitatively identical with that of virginal protein, but the amplitude of the 273-nm peak is reduced. The different CD spectrum obtained by subtracting the spectrum of virginal elastase from that of the enzyme with ten groups acetylated is shown in the inset of Fig. 5. It is found to be maximal at 274 nm and has great similarity to the CD spectrum of *N*-acetyltyrosine amide in the same wavelength region (29). In the middle uv region, acetylation to an extent greater than seven groups (nine or ten tyrosines acetylated) results in a small increase in negative ellipticity between 220 and 232 nm, strongly suggesting tyrosine contribution to the spectral details observed in this region.

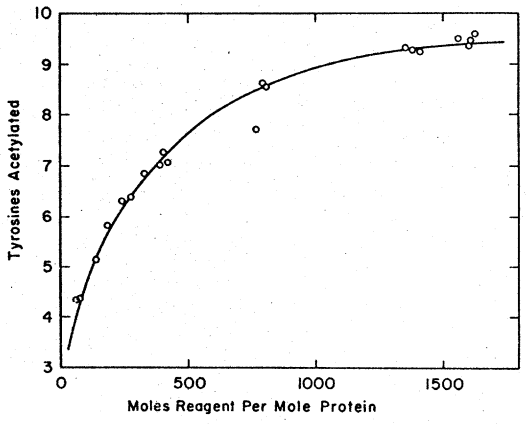

FIG. 4. Dependence of elastase tyrosine acetylation on the concentration of *N*-acetylimidazole; 0.05 M borate buffer (pH 5) 4°C, protein concentration: 0.7–0.8 mg/ml.

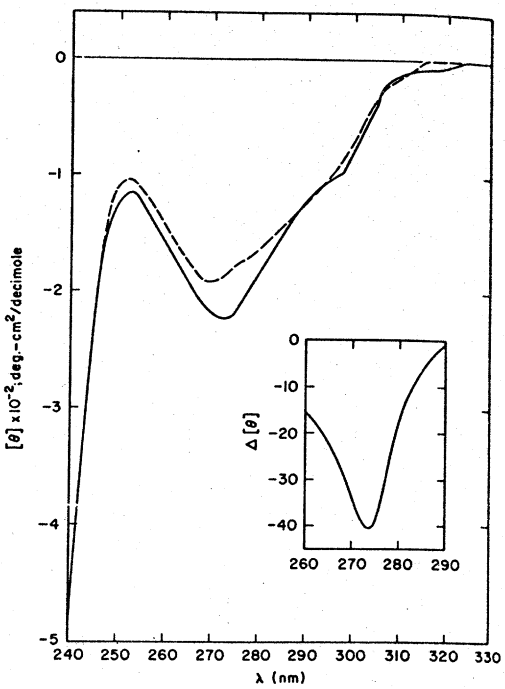

FIG. 5. Near-uv CD spectra of native elastase and elastase acetylated to the extent of ten tyrosines (average of three experiments); 0.01 M acetate buffer (pH 5) 2°C. Inset: difference circular dichroism spectrum between virginal and acetylated elastase.

Deacetylation

N-acetylimidazole can also acetylate amino acids other than tyrosine. Acetylated

elastase was, therefore, treated with 2 M hydroxylamine to determine the number of acetyl groups which had been introduced. This was done at pH 11.6 to establish the total number of labile acetyls (25) and also at pH 7.0 to determine specifically those attached to tyrosine residues (22). It was found that the number of acetyl residues removed at pH 7.0 was in good agreement with that of reacted tyrosines, as determined spectrophotometrically. This agreement justified the use of $\Delta\epsilon_{278} = 1160$ for tyrosine (21) in this particular case. Indiscriminate use of this value has been seriously questioned (30).

The total number of labile acetyl residues which could be removed at pH 11.6 was found to range between 6 and 17. Thus, acetylation of four to six tyrosines is accompanied by the acetylation of two to four other residues. Just above this level of tyrosine acetylation, extraneous acetylation jumps to six groups and does not exceed ten residues at a reagent level at which ten tyrosines are modified.

Cyanuration

All cyanuration experiments were carried out at 4°C due to the lability of elastase at room temperature. Preliminary experiments were carried out at a CyF concentration of 0.0232 M at pH 9.5, 10.0, 10.5, 11.0, 12.0 and 12.5. The average numbers of tyrosines reacted at these conditions were: 5.0 at pH 9.5; 6.1 at pH 10.0; 6.8 at pH 10.5; 7.5 at pH 11.0; 8.1 at pH 12.0 and 8.3 at pH 12.5. On the basis of these results, concentration curves were obtained at pH 9.5, 10.0, 10.5, 11.0 and 12.5. A plateau was obtained only at pH 11.0 at a level of eight reacted groups. At all other pH values the results were poorly reproducible and asymptotic in nature, tending toward eight groups below pH 11 and becoming more than eight at pH 12.5. This suggests approach-to-plateau conditions. It must be recalled that the cyanuration reaction requires that the tyrosine hydroxyl be ionized. Thus, the lack of appearance of a plateau below pH 11 does not necessarily indicate that the reacted groups are different in nature, but might simply reflect the competition between tyrosine cyanuration and decomposition of the reagent (31).

DISCUSSION

The above-described results indicate that the tyrosine residues of elastase exist in nonidentical states. The titration experiments show that ten groups are essentially equally accessible to hydroxyl ions, while one residue remains deeply buried even at pH 12.5, at which value the enzyme is already highly denatured. This is in excellent agreement with the X-ray crystallographic results, on the basis of which ten out of the 11 tyrosine residues have been classified as external side chains, with the eleventh residue (Tyr 234) as buried (1, 2).

While accessibility to hydroxyl ions shows that the phenolic hydroxyls of ten tyrosines are in contact with solvent and not strongly hindered either by strong electrostatic interactions with neighboring groups or by hydrogen bond formation, chemical modification indicates a considerable gradation in tyrosine reactivity. The CyF modification results give one plateau of eight residues at pH 11.0. At low temperature, the conformational state at pH 11.0 is not significantly different from that of the native enzyme, since the far uv CD spectrum is identical at pH 7 and 10.9. Besides, at pH 11 and 4°C the enzyme is still fully active. Thus, this level of reactivity with CyF can be equated to that of the native enzyme. Furthermore, Kaplan *et al.* (14) have shown unequivocally the integrity of elastase structure at pH 10.5 and 10°C.

The results of acetylation with *N*-acetylimidazole (Fig. 4) indicate that, on the basis of the reactivity of tyrosine residues toward this reagent, these can be subdivided into classes containing 7–8, 2–1, one and one residues each, in order of decreasing reactivity.

Thus, if one assumes that chemical modification affects the same residues in both cases, there are eight residues which are easily reactive at their hydroxyl group. Furthermore, there are two more *N*-acetylimidazole-reactive, but CyF-unreactive residues, and one totally unreactive tyrosine. This gradation in reactivity may represent either accessibility of groups to the reagents,

if the problem is analyzed strictly in terms of space, or differences in microenvironment, such as polarity, hydrogen bonding or charge distribution (30, 32). In fact, it must be remembered that chemical modification reactions are a strong function of competition between reaction with the protein residue and reagent decomposition (31). From a comparison of the cyanuration and acetylation reactions, it may be inferred that the two *N*-acetylimidazole less reactive groups (unreactive toward CyF) are actually located in more sterically hindered microenvironments than the other groups, since the spacial requirements of the cyanuration reaction intermediate are more constricting.

The high degree of accessibility of tyrosine residues to chemical modifiers is in good agreement with the results of the solvent perturbation studies in which it was reported that tyrosine residues are exposed to glycerol to the extent of 45 % (27). Since glycerol is a large perturbant (33), this figure must represent a lower limit of exposure. Therefore, in terms of partial exposure, this result could well mean that the majority of the residues are accessible to the solvent to various degrees. Thus, the classification into three classes of reactive (8 + 1 + 1) and one buried residue is fully consistent with the reported exposure to glycerol.

Comparison of the CD spectra of acetylated and native elastase shows that the acetylation of seven tyrosine hydroxyls has no effect whatever on any of the spectral regions. This indicates that these groups have at most weakly optically active transitions, suggesting that they possess a fair degree of rotational freedom in the native enzyme or are not located in an asymmetric environment (34, 35).

Although acetylation of the next three groups (ten tyrosines acetylated) again results in no changes in the far uv CD spectrum, indicating the maintenance of the native secondary structure, this reaction does bring on subtle changes both at 273 and between 220 and 232 nm. These changes permit assignment of at least part of the band intensities in these regions to the transitions of the three less reactive tyrosines.

Taking the two spectral regions in turn, there are no changes in optical activity between pH 5 and 8.5 which could account for the reported 8°-change in optical rotation at 365 nm between pH 7.5 and 9 (12). Above pH 8.5, the near uv CD spectrum undergoes a gradual transition. This spectrum, which is totally different from that of chymotrypsin, is strikingly similar to that of ribonuclease (34, 36, 37) and that of trypsin (38). By analogy, it may be inferred that, contrary to the case of the chymotrypsins where tryptophans make strong contributions to the spectrum (28, 35), the principal contributions in elastase stem from tyrosine and disulfide transitions. Thus, the shoulder at 310–320 nm is probably due to disulfides (39, 40), while that at 295 nm may represent the only discernible contribution of typtophans (34, 35). The 273-nm band probably contains more disulfide than tyrosine contribution. The acetylation experiments show that seven tyrosines are not optically active in this region. Therefore, the small change at 273 nm in titration from pH 8.5 to 10.6 must reflect the ionization of the three less reactive residues. Their possession of optical activity suggests restraint of freedom of rotation and is consistent with their lowered reactivity toward acetylation and lack of reactivity toward cyanuration. The change between 290 and 310 nm most probably results from perturbation of disulfide residues, which becomes considerable at pH 10.9. The strong increase in negative ellipticity below 260 nm reflects spectral changes at lower wavelengths, in particular, in the 240-nm region.

In the 230–250-nm region, spectral changes occur only above pH 9.5. While superficially both the CD spectra and their changes appear similar to those of the chymotrypsins (9, 34), in actuality they are grossly different. First, the position of the negative apex in native elastase (234 nm) is displaced to the red relative to chymotrypsin (230 nm) and the negative minimum (232 nm in elastase, 224 nm in chymotrypsin) is much narrower in elastase. Second, the intensity of the peak is much weaker in elastase than in chymotrypsin, this spectral feature in elastase being much more remi-

niscent of that found in chymotrypsinogen (9, 34). Third, and most significant, raising of the pH from 7 to 10.5, results in spectral changes in both enzymes; in elastase the negative peak is shifted to the red (from 234 to 238 nm); in chymotrypsin its position remains invariant, and only its intensity changes. This red shift in elastase on raising the pH leads to an assignment of the CD features between 220 and 240 nm to predominantly tyrosine transitions. The essential disappearance of spectral details at pH 11.3 reflects denaturation of the enzyme (see Table 1) and suggests that the buried tyrosine makes no contribution to the spectrum in this region, since from the titration results it is known to be still highly constrained at pH 12.5.

A suggestion has been made that the conformational stability of elastase is controlled by the phenolic hydroxyl of a tyrosine residue (13). Since acetylation of the ten "surface" tyrosines affects neither the secondary structure nor the activity of the enzyme, these may be ruled out. The possibility that this is a surface residue can, therefore, be discounted. The reported loss of activity incurred during the alleged modification of a single tyrosine with TNBS (13) can be caused then only by the general disruption of the tertiary structure by the introduction of the bulky group into some position on the protein. Kaplan *et al.* (14) have proposed that a general structural transition is triggered by the ionization of a group with a $pK_a > 11$. It is not likely that this group is a tyrosine, since ten titrate normally, while the eleventh is still unionized at pH 12.5. Since at the pH the enzyme is already denatured, one might infer that this group (Tyr 234) is located within a core which is highly resistant to denaturation. Other possibilities could be an arginine or an abnormally titrating lysine residue. An alternate possibility is the disruption of the protein by general nonspecific electrostatic repulsion (41), as it assumes a progressively increasing net negative charge. Such cooperative transitions are typical of protein denaturation.

In conclusion, it can be stated that the similarities between chymotrypsin and elastase cannot be extended to the alkaline transitions. The change with pH of the 234-nm CD band in elastase most probably reflects the ionization of some somewhat constrained tyrosine residues without a concomitant conformational change. The similar CD change in chymotrypsin is definitely linked to the migration of Asp-194 from Ile-16 to His-40 (34, 42).[4] Furthermore, in chymotrypsin, this is accompanied by a large change in the far uv CD spectrum,[4] which in elastase remains strikingly invariant between pH 5 and 11.

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REFERENCES

1. Shotton, D. M., and Watson, H. C. (1970) *Nature* **225**, 811.
2. Shotton, D. M., and Hartley, B. S. (1970) *Nature* **225**, 802.
3. Watson, H. C., Shotton, D. M., Cox, J. M., and Muirhead, H. (1970) *Nature* **225**, 806.
4. Bender, M. L., and Marshall, T. H. (1968) *J. Amer. Chem. Soc.* **90**, 201.
5. Shotton, D. M., and Watson, H. C. (1970) *Phil. Trans. Roy. Soc. London*, **B257**, 111.
6. Hartley, B. S. (1970) *Phil. Trans. Roy. Soc. London*, **B257**, 77.
7. Fersht, A. R. (1972) *J. Mol. Biol.* **64**, 497.
8. Oppenheimer, H. L., Labouesse, B., and Hess, G. P. (1966) *J. Biol. Chem.* **241**, 2720.
9. Hess, G. P., McConn, J., Ku, E., and McConkey, G. (1970) *Phil. Trans. Roy. Soc. London*, **B257**, 89.
10. Kaplan, H., and Dugas, H. (1969) *Biochem. Biophys. Res. Commun.* **34**, 681.
11. Gertler, A., and Hofmann, T. (1970) *Can. J. Biochem.* **48**, 384.
12. Kaplan, H., Symonds, V. B., Dugas, H., and Whitaker, D. R. (1970) *Can. J. Biochem.* **48**, 649.
13. Rao, L., and Hofmann, T. (1970) *Can. J. Biochem.* **48**, 1249.
14. Kaplan, H., Stevenson, K. J., and Hartley, B. S. (1971) *Biochem. J.* **124**, 289.

[4] M. J. Gorbunoff and S. N. Timasheff, unpublished results.

15. SHOTTON, D. M. (1970) *in* Methods in Enzymology (Perlman, G. E., and Lorand, L., eds.), Vol. XIX, p. 113, Academic Press, New York.
16. GORBUNOFF, M. J. (1969) *Biochemistry* **8**, 2591.
17. WETLAUFER, D. B. (1962) *Advan. Protein Chem.* **17**, 303.
18. CRAMMER, J. L., AND NEUBERGER, A. (1943) *Biochem. J.* **37**, 302.
19. SAGE, M. J., AND SINGER, S. J. (1962) *Biochemistry* **1**, 305.
20. GORBUNOFF, M. J. (1972) *in* Methods in Enzymology (Hirs, C. H. W., and Timasheff, S. N., eds.), Vol. XXV, p. 506, Academic Press, New York.
21. RIORDAN, J. F., WACKER, W. E. C., AND VALLEE, B. L. (1965) *Biochemistry* **4**, 1758.
22. HESTRIN, S. (1949) *J. Biol. Chem.* **180**, 249.
23. BENDER, M. L., BEGUE-CANTON, M. L., BLAKELEY, R. L., BRUBACHER, L. J., FEDER J., GUNTER, C. R., KEZDY, F. J., KILLHEFFER, J. V., JR., MARSHALL, T. H., MILLER, C. G., ROESKE, R. W., AND STOOPS, J. K. (1966) *J. Amer. Chem. Soc.* **88**, 5890.
24. WASI, S., AND HOFMANN, T. (1968) *Biochem. J.* **106**, 926.
25. BALLS, A. K., AND WOOD, H. N. (1956) *J. Biol. Chem.* **219**, 245.
26. LEWIS, U. T., WILLIAMS, D. E., AND BRINK, N. G. (1956) *J. Biol. Chem.* **222**, 705.
27. VISSER, L., AND BLOUT, E. R. (1971) *Biochemistry* **10**, 743.
28. GORBUNOFF, M. J. (1971) *Biochemistry* **10**, 250.
29. ETTINGER, M. J., AND TIMASHEFF, S. N. (1971) *Biochemistry* **10**, 831.
30. MYERS, B., II, AND GLASER, A. N. (1971) *J. Biol. Chem.* **246**, 412.
31. GORBUNOFF, M. J. (1972) *Biopolymers*, in press.
32. GORBUNOFF, M. J. (1970) *Arch. Biochem. Biophys.* **138**, 684.
33. LASKOWSKI, M., JR. (1966) *Fed. Proc.* **25**, 20.
34. TIMASHEFF, S. N. (1970) *in* The Enzymes (Boyer, P., ed.), Vol. 2, p. 371, Academic Press, New York.
35. STRICKLAND, E. H., HORWITZ, J., AND BILLUPS, C. (1969) *Biochemistry* **8**, 3205.
36. PFLUMM, M. N., AND BEYCHOK, S. (1969) *J. Biol. Chem.* **244**, 3973.
37. SIMONS, E. R., SCHNEIDER, E. G., AND BLOUT, E. R. (1969) *J. Biol. Chem.* **244**, 4023.
38. PANTALONI, D., D'ALBIS, A., AND DESSEN, P. (1968) *J. Chim. Phys.* **65**, 196.
39. BEYCHOK, S. (1965) *Proc. Nat. Acad. Sci. U. S.* **53**, 999.
40. BEYCHOK, S. (1967) *in* Poly-α-Amino Acids (Fasman, G. D., ed.), p. 293, Marcel Dekker, New York.
41. TIMASHEFF, S. N. (1970) *in* Biological Polyelectrolytes (Veis, A., ed.), p. 1, Marcel Dekker, New York.
42. FREER, S. T., KRAUT, J., ROBERTUS, J. D., WRIGHT, H. T., AND XUONG, NG. H. (1970) *Biochemistry* **9**, 1967.